Quantitative method to study the network formation of endothelial cells in response to tumor angiogenic factors

F. Amyot, K. Camphausen, A. Siavosh, D. Sackett and A. Gandjbakhche

Abstract: To study the network formation of endothelial cells (ECs) in an extracellular matrix (ECM) environment, we have devised an EC aggregation-type model based on a diffusion limited cluster aggregation model (DLCA), where clusters of particles diffuse and stick together upon contact. We use this model to quantify EC differentiation into cord-like structures by comparing experimental and simulation data. Approximations made with the DLCA model, when combined with experimental kinetics and cell concentration results, not only allow us to quantify cell differentiation by a pseudo diffusion coefficient, but also measure the effects of tumor angiogenic factors (TAFs) on the formation of cord-like structures by ECs. We have tested our model by using an *in vitro* assay, where we record EC aggregation by analysing time-lapse images that provide us with the evolution of the fractal dimension measure through time. We performed these experiments for various cell concentrations and TAFs (e.g. EVG, FGF-b, and VEGF). During the first six hours of an experiment, ECs aggregate quickly. The value of the measured fractal dimension decreases with time until reaching an asymptotic value that depends solely on the EC concentration. In contrast, the kinetics depend on the nature of TAFs. The experimental and simulation results correlate with each other in regards to the fractal dimension and kinetics, allowing us to quantify the influence of each TAF by a pseudo diffusion coefficient. We have shown that the shape, kinetic aggregation, and fractal dimension of the EC aggregates fit into an *in vitro* model capable of reproducing the first stage of angiogenesis. We conclude that the DLCA model, combined with experimental results, is a highly effective assay for the quantification of the kinetics and network characteristics of ECs embedded in ECM proteins. Finally, we present a new method that can be used for studying the effect of angiogenic drugs in *in vitro* assays.

1 Introduction

Angiogenesis, the transient formation of new blood vessels, is mainly observed under certain physiological conditions in the adult (e.g. oestrous cycle, placenta, or wound healing). Deregulated angiogenesis has been implicated in diseases such as retinopathies, rheumatoid arthritis, and cancer [1]. Although recent *in vitro* models have been crucial in determining the effects of growth factors and inhibitors on cell migration and sprouting, the fundamental processes of cell adhesion kinetics during tumor induced angiogenesis have not been quantified. During early stages of angiogenesis, endothelial cells (ECs) migrate from pre-existing vessels through the extracellular matrix (ECM), adhere to each other, and form a specific pattern [2]. Following this initial event, an accumulation of ECs form new, small finger-like capillaries [3]. The sprouts grow in length with the migration and further recruitment of ECs, and move towards the tumor.

Efforts to understand the mechanism of angiogenesis has lead to the development of many *in vivo* and *in vitro* models that explore the fundamental role of ECs: proliferation, migration, and intercellular adhesion. Although there is clear experimental evidence that disrupting the tumor angiogenic factor (TAF) cell receptors has a direct effect on the final structure of the capillary network [4], it is unclear how cells or the TAF concentration can affect EC pattern formation. In order to investigate EC adhesion, methods (i.e. assays) have been developed to observe and quantify the movement of cells under defined *in vitro* conditions. The underlying principle of these assays is to culture ECs on biogels and determine how ECs mimic the *in vivo* early stage of angiogenesis, i.e. cell aggregation into cord-like structures [5–7]. However, there is not a quantitative method to describe the transformation of ECs into cord-like structures over time.

Pattern formation in a non-equilibrium system typically occurs when one phase grows at the expense of others. Examples of such pattern-forming processes are biological flocs from bacteria [8], the flocculation of colloids [9], and the growth of a crystal [10]. This type of phase change requires the transport of at least one conserved quantity via diffusion. Similar growth processes occur with colloids, coagulated aerosols, and electrochemical deposition [11]. The aggregates formed in these cases have extreme

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multi-branching, are agglomerated, and possess dendrites, all of which are typical of fractal patterns. Witten and Sander [12, 13] have described such growth by a diffusion limited aggregation model. In addition, Meakin [14] and Kolb *et al.* [15] developed related models in which clusters of particles diffuse, and clusters of all sizes stick together upon contact. These models indicate that the obtained structures have remarkable scaling and universality properties: they are fractal.

In this paper, an aggregation-type model of endothelial cells is studied based on a diffusion limited cluster aggregation (DLCA) model. DLCA is an effective model because it exhibits the same fractal pattern (multi-branching and dendrites) as EC cord-like structures. We compare the kinetics and fractal dimensions measured *in vitro* to simulations for different cell and TAF concentrations. Finally, we show from *in vitro* experiments and a simulation, that the DLCA model is well adapted for modelling the first stage of angiogenesis. The shape, aggregation, and fractal dimension of the clusters fit into an *in vitro* model that is able to reproduce the early stage of angiogenesis. These studies present a quantitative model for characterising the effect of TAFs as well as any other stimulus on angiogenesis.

2 Experiment

2.1 Materials and methods

2.1.1 Cell culture: Human umbilical vein endothelial cells (HUVECs) were provided by the American Type Culture Collection (ATCC) and used in all experiments. The cells were grown as a monolayer in 75 mm dishes with endothelial cell growth medium (EGM2, Clonetics). The media contained 2% fetal bovine serum and a complement of growth factors. Cultures were maintained at 37°C and 5% CO_2.

2.1.2 In vitro angiogenesis assay: For the *in vitro* angiogenesis assay, the endothelial cells were seeded on a 13 × 35 mm^2 glass cover slip previously coated with a gel at 4°C. The gel was a basement membrane extract (Trevigen, Gaithersburg, Maryland). This gel (300 µl) was allowed to polymerise for 1 h at 37°C before the addition of the media and cells. The cells were suspended in culture media and 500 µl of this solution was added on top of the gel.

We labelled the cell with a green fluorescent marker (SIGMA PKH2-GL). To observe cell migration, we placed the glass cover slip in a closed live-cell chamber (FCS2, Biopthechs), which allowed for a safely maintained temperature-controlled environment. The temperature was maintained at 37°C during the entire assay.

2.1.3 Images of the network growth: An image analysis system was used to measure the geometrical properties of the endothelial cell aggregates. Cord-like structure formation occurs in three stages: (i) migration and early network formation; (ii) network remodelling; and (iii) differentiation of tubular structures [5]. To analyse early network formation, ECs were plated on a basement membrane film. Cell migration and aggregation were recorded using time-lapse video microscopy. The image analysis system consisted of a confocal microscope (Olympus) camera. Images were recorded every hour at 4× magnification. The pictures were recorded at regular intervals for 10 h. Individual cells, cluster trajectories, and pattern formation were tracked using computer-assisted analysis. Statistical time analysis of the fractal dimension of the cell pattern was performed and the fractal dimension was calculated using the box method with Matlab® software.

2.2 Experimental results

Figure 1 shows several steps of cell aggregation. Before forming a cord-like structure pattern, cells move and adhere to each other. The media and gel were homogeneous and there was no growth factor gradient. The chemotactic response of the cells in a specific direction due to external stimuli was limited; the cells moved by random walk.

After 2 h, randomly seeded ECs start moving, interacting, and adhering to their neighbours, and start to form a continuous multicellular network. Despite the fact that individual cells folded up to form capillary-like tubes, the geometry was not substantially modified after 4 h. Since the main geometrical features of the network patterns were determined during the first stage of the process, the subsequent experimental and theoretical approaches of this study focused on the early stage of EC migration.

Growing fractal structures develop more detail over time, and can be characterised with fractal dimension. At any given point in development, the growing structure will occupy a finite surface, which determines the fractal dimension of the object. Here, the fractal dimension is calculated by the box method and is given by the following relation: $N(\ell) \sim \ell^{-Df}$, where $N(\ell)$ is the number of boxes of side ℓ required to cover the structure contained in the space of size L. The power of this relation gives the fractal dimension (Df).

Our results show that as the size of the aggregates increases with time, the fractal dimension of the aggregate decreases. Furthermore, several experiments were devised

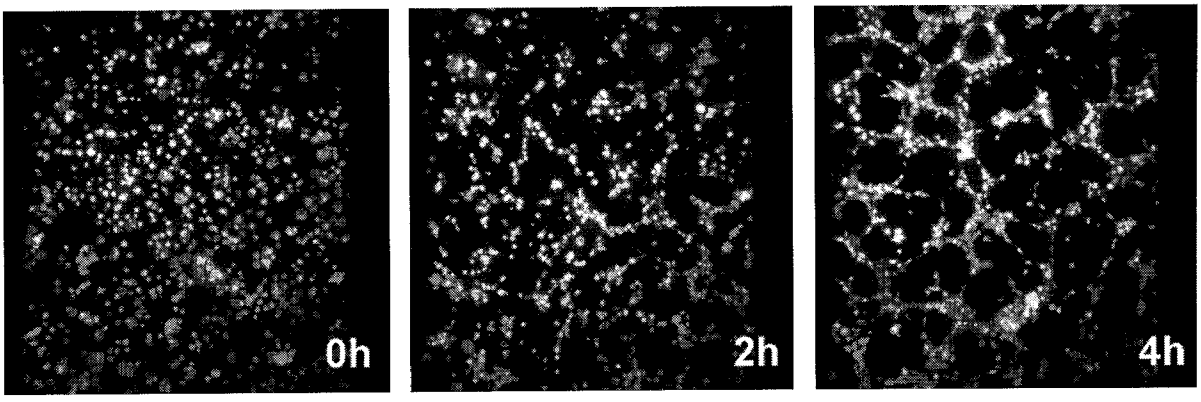

Fig. 1 *Aggregation of endothelial cells at three different time points (0 h, 2 h, and 4 h). The cells aggregate and begin to form a pattern. ECs (HUVECs) were spread on a basement membrane gel*

in order to evaluate the affect of different factors on cell aggregation: cell concentration and presence of different TAFs (e.g. EGF, FGF, and VEGF).

Figure 2 shows the time evolution of the fractal dimension measured by the box method for different initial cell concentrations. The initial quantity of cells strongly influences the values of the initial and final fractal dimensions as well as the kinetics. The cord-like structure formed faster with a higher cell concentration. However, this phenomenon was limited. Small quantities of cells did not aggregate; the cells were unable to form a tube network. In contrast, a high cell population fully aggregated, forming a film instead of a cord-like structure or fractal object.

Capillary tube formation by ECs is the result of a complex process. Several TAFs need to be available to allow or accelerate this process [16]. These growth factors influence cell aggregation kinetics and final fractal dimension. Figures 3 and 4 show the aggregation kinetics for the same cell concentration exposed to different TAFs. Figure 3 shows that the presence of FGF in the culture media increases the tube formation kinetics, i.e. the network is formed quicker. Similarly, the presence of VEGF provokes an aggregation of the ECs, but slower than the media containing all the TAFs. We did not see aggregation for the cells treated with media lacking TAF after 8 h of recording.

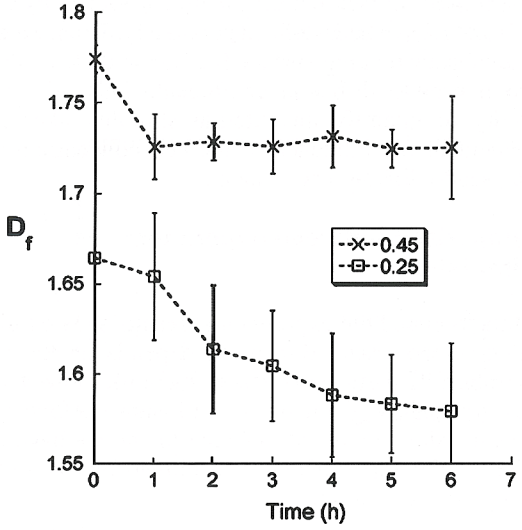

Fig. 2 *Cord-like networks formed by plating two different cell concentrations on basement membrane gels, with surface fractions of 0.25 and 0.45. The fractal dimension (Df) measurements show us the kinetics of cord-like structure formation through time. Network formation is quicker with a higher concentration of cells*

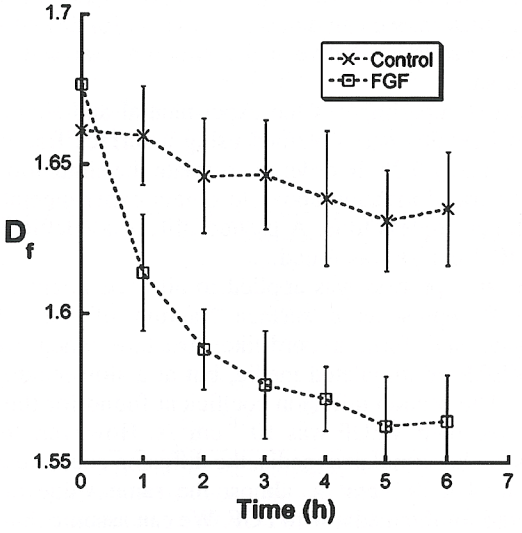

Fig. 3 *Endothelial cells were spread on a basement membrane gel with FGF (control does not have FGF). The evolution of the fractal dimension was measured with a cell surface fraction of 0.25. The presence of FGF increases the ECs transformation into cord-like structures*

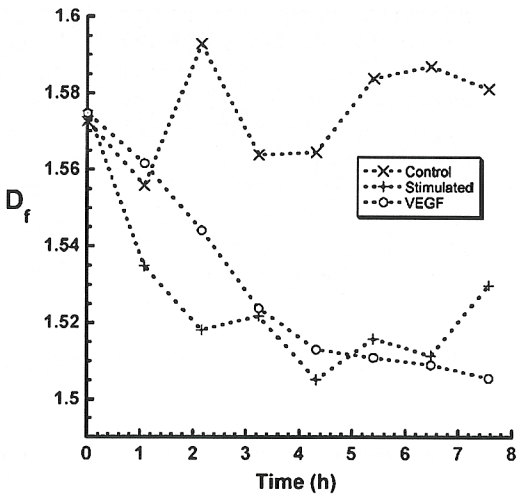

Fig. 4 *Endothelial cells with a surface fraction of 0.21 were spread on basement membrane gel. Three kinds of media were added: control media (without TAF), media with VEGF and media with both FGF and VEGF (stimulated). The fractal dimension measure of the cell network over time shows that the aggregation kinetics of the stimulated media is quicker than the media with VEGF. In addition, the kinetics of aggregation are quicker for media including VEGF than media without TAF*

3 Simulation

The two-dimensional simulations used a simple square domain with periodic boundary conditions. At the beginning of the simulation, a surface fraction (ϕ) of the domain was considered at random and occupied. The number of particles (N) with radius R and square box of size $L(L = 1)$ defines the surface fraction:

$$\phi = \frac{N.\pi R^2}{L^2}$$

Two particles separated by a distance of $2R$ belonged to the same cluster. Each cluster or single particle moved in all directions by diffusion, where the diffusion coefficient was inversely proportional to the size of the cluster.

The study domain is a box of size L and the simulations were performed with a surface fraction contained between 0.08 and 0.53. The radius (R) of the particles was 0.0075. This value is the same as the experimental ratio measured between the observation window and the cell radius.

In the off-lattice algorithm used in this simulation, circular particles with radius R were initially randomly distributed in a square box without particle overlapping. Thus, at the beginning of the time course, particles were unaggregated. The movement of the cluster consisted of steps of length $d = \sqrt{Dt}$ in a randomly selected direction. If two particles, or two clusters, are closer than a distance $2R$, then we consider that these two clusters have aggregated together and formed a new cluster.

The value of cluster size changes with time, affecting the value of the cluster diffusion coefficient. Thus, we assume

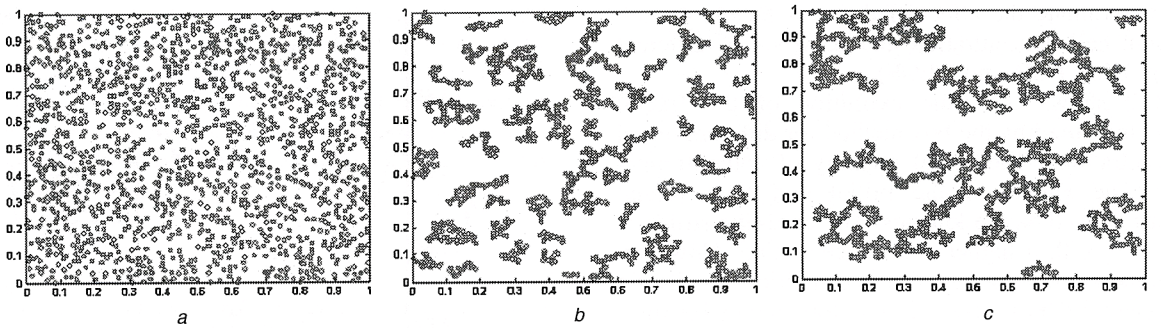

Fig. 5 *Cluster growth by simulation. The three situations correspond to times*

a $t = 0$
b $t = 1.4$
c $t = 10$
The simulation was carried out for 1500 particles (□ö = 0.26, each with a radius of 0.0075 and initially randomly spread) and the time is dimensionless

the cluster diffusion coefficient is inversely proportional to the number of particles in the cluster.

Several simulations were performed and in each simulation, D and L were dimensionless and equal to one. Figure 5 shows the steps involved in a simulation with a ϕ of 0.26 (1500 particles).

At each step, we can calculate a fractal dimension and then simulate the fractal dimension evolution of these clusters. Like the experimental approach, the clusters' fractal dimensions were obtained from the box method. Each curve is the average of 25 simulations.

Figure 6 gives the initial and final fractal dimension values for several surface fractions. As the particle concentration increases, the clusters' fractal dimension increases slightly, making the clusters more compact. This was expected for low particle concentrations because when two clusters touch and merge mainly at their tips, then the cluster formed is opened. For high concentrations, the clusters are close together, making it easier for them to touch in the middle of the cluster instead of at the tips. Moreover, for very high concentrations, the percolation threshold is reached. The curve reaches a threshold, the percolation limit, for a maximum packing value ($\phi \sim 0.57$).

The fractal dimension decreases exponentially to reach a threshold value. This value, like the initial value, increases with particle concentration. The characteristic time also changes with the fraction surface, even if it keeps the same

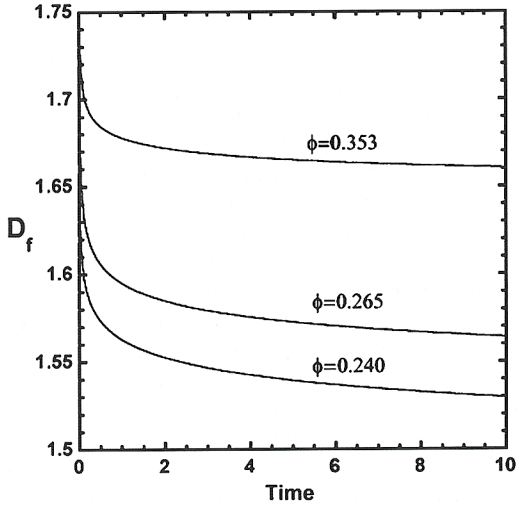

Fig. 6 *Simulated evolution of fractal dimensions (Df) for different particle populations or surface fractions (Φ). Time is dimensionless. An increase in the fraction surface leads to quicker kinetics*

order of magnitude. However, for the same fraction surface, the characteristic time is also given by the diffusion coefficient used. In fact, the time is dimensionless by D/L^2, where D and L have been defined previously. Thus, we could create an abacus relating surface fraction and characteristic time to a pseudo diffusion coefficient. This allowed us the ability to quantify an external stimulus like TAFs, independent of the influence of the cell concentration, by using a pseudo diffusion coefficient.

4 Comparison between experiment and simulation

The simulation and the experimental results showed us that the value of the fractal dimension depends on the initial concentration of particles (surface fraction). However, the kinetics are mainly given by the diffusion coefficient. To compare the simulation and the experiment, we have made the time for the experimental data dimensionless with the following transformation:

$$t^* = t \times \frac{L^2}{D}$$

where t is the experimental time, L is the length of the experimental observation window ($L = 0.12\,\text{cm}$). D represents the pseudo diffusion coefficient which characterises each growth factor. Therefore, for a particular value of surface fraction, we can determine a value for D that gives the best correlation between the experimental and simulation data.

Figure 7 superimposes the experimental and simulation data. All results were obtained using a surface fraction of approximately 0.25. In order to correlate the simulation, we used the transformation above to obtain experimental data with dimensionless time. A pseudo diffusion coefficient of $3.5 \times 10^{-6}\,\text{cm}^2/\text{s}$ was found.

The same process was applied to all experimental data. Figure 8 represents a surface fraction of 0.21. Cells aggregate and form a cord-like structure when treated with VEGF or stimulated media, but at a slower rate with VEGF. The pseudo diffusion coefficient found for the cells stimulated with VEGF was $10^{-6}\,\text{cm}^2/\text{s}$. However, for the cells with the stimulated media, the diffusion coefficient was equal to $4 \times 10^{-6}\,\text{cm}^2/\text{s}$, almost the same value as that measured for the media with FGF. We can assume that FGF plays a major role during aggregation and cord-like network formation. This method allows us to quantify the influence of a specific TAF independently of cell concentration. Table 1 shows how we can compare the influence of each TAF despite different surface fraction values. For example,

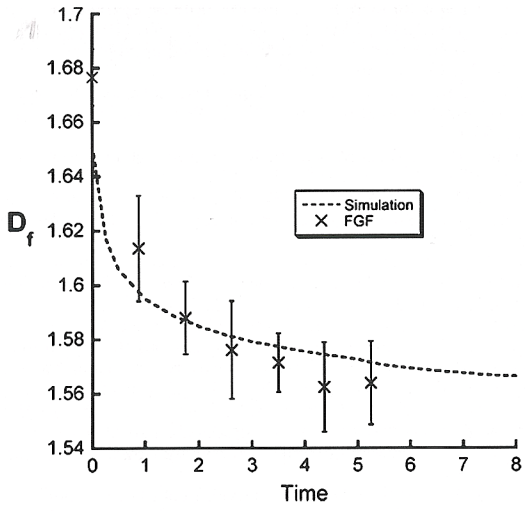

Fig. 7 *The experimental fractal dimension measured with a cell surface fraction of 0.45 is compared to the simulation data made with the same surface fraction. In order to compare the two curves, the time is dimensionless by D/L^2. The number found for D is equal to 3.5×10^{-6} cm^2/s*

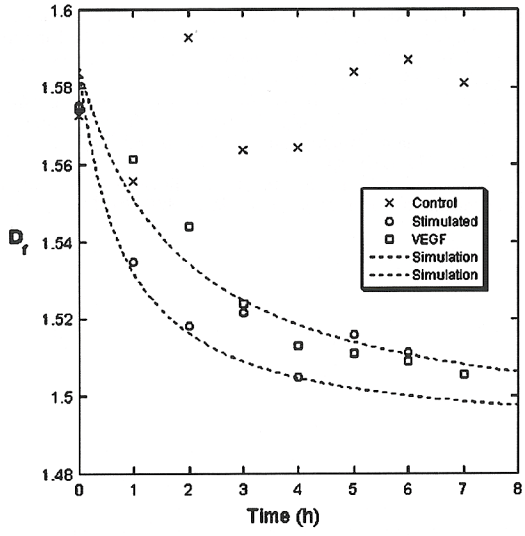

Fig. 8 *Endothelial cells, corresponding to a fraction surface of 0.21, were spread on a gel in the presence of VEGF, all the TAF (stimulated) and without TAF (control). In order to compare the experimental data to the simulation, the simulation data was made dimensionless by D/L^2. Here, the diffusion coefficient for the cells in stimulated media is equal to 4×10^{-6} cm^2/s while the diffusion coefficient for cells in media with VEGF is 10^{+6} cm^2/s*

Table 1: Comparison of influence of each TAF

Growth factor	Half-time (h)	D
FGF	0.95 ($\phi = 0.45$)	3.5×10^{-6} cm^2/s
VEGF	2.19 ($\phi = 0.21$)	1×10^{-6} cm^2/s
Stimulated	0.87 ($\phi = 0.45$)	4×10^{-4} cm^2/s
	1.79 ($\phi = 0.25$)	

The fractal dimension of cord-like structure formation is measured for cells in the presence of FGF, VEGF, and with different kinds of TAFs. The half-time value represents the time at which the fractal dimension is equal to the mean value between the fractal dimension at $t = 0$ and $t = 8$ h. ϕ represents the surface fraction measured for each experiment. D represents the diffusion coefficient obtained from the transformation used from the fractal dimension values. This coefficient is independent of the cell surface fraction.

cells in media containing all the TAFs form the cord-like structure twice as fast with a surface fraction equal to 0.45 than with a surface fraction of 0.25. This prevents us from comparing the effect of two TAFs for two populations of cells. In fact, with the stimulated media with FGF and a surface fraction of 0.25, the cord-like structure appears later than with the FGF media and a surface fraction of 0.45. In this paper, we propose a special transformation which releases us from the constraints of using a single surface fraction. This transformation allows us to compare two kinetics, even if the surface fractions are different.

5 Discussion

In recent years, studies have been developed to quantify various aspects of vascular morphogenesis and angiogenesis that propose novel *in vitro* cell culture models in order to reproduce the definitive elements of angiogenesis. Several such effective models include measuring the length of cords in a network [17–18], the number of nodes in capillary-like structures [19], and the radius of cell invasion in a collagen matrix [20]. In order to characterise the network complexity of the ECs after aggregation and cord-like pattern formation, measurements such as cord length and number of nodes have proven very useful. For our experiments, we prefer to use the fractal dimension measurement as an elegant way to follow the transformation of each individual cell into complex cord-like structures. While the length of cords and number of nodes provide information about the complexity of the network after it is established, the fractal dimension measurement allows for the precise tracking of the changes in morphology throughout the experiment.

With the introduction of the methods described above, more has been elucidated about the effect of angiogenic factors on individual cells, where information is obtained about the migration and sprouting of each EC. In our experiments, we have successfully tracked the interaction of multiple cells in cell adhesion processes leading to the formation of cord-like structures. In addition, the short duration of our experiments, which do not exceed 8 h, serves as an advantage for quantifying the effects of growth factors on cell adhesion and cell migration because the possibility of cell proliferation during an experiment is reduced.

The study can also be used to quantify the influence of different stimuli on angiogenesis formation. For example, the same study could be used to determine the effects of gel composition, viscosity, and thickness, which are activating factors for angiogenesis [21]. In fact, the aggregation of endothelial cells into cord-like structures is the result of different factors in the cellular environment. Ingber [22] has shown that stress induced by the micro-environment in the vicinity of the cell is also an angiogenic factor. Recent studies have shown how ECs integrate shear stress and induce a morphological response, i.e. reorganisation of the cell into a cord-like structure [23–25].

6 Conclusions

This study has shown that a simple model of DLCA can explain the specific pattern created by the auto-organisation process performed by ECs. The shape, kinetic aggregation, and fractal dimension of the EC aggregates fit into an *in vitro* model that is able to reproduce the first stage of angiogenesis. The model appears to be successful in describing cell network assembly within two parameters, the cell surface fraction and the number of cells. The model can also quantify EC response to external stimuli. In addition, the model describes the early stage of

angiogenesis, which gives the pattern of the future vascular network. The model can also quantify EC response to angiogenic activators and inhibitors in drug studies as well as other external stimuli. In the future, we would like to expand our model to study complex biological parameters such as ECM heterogeneity, ECM-cell interactions, and cell-cell adhesions.

7 References

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